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Rapid Polymerase Chain Reaction-Based Detection of Epidermal Growth Factor Receptor Gene Mutations in Lung Adenocarcinomas

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Somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (*EGFR*) gene are present in lung adenocarcinomas that respond to the EGFR inhibitors gefitinib and erlotinib. Two types of mutations account for ~90% of mutated cases: short in-frame deletions in exon 19 and a specific point mutation in exon 21 at codon 858 (L858R). Screening for these mutations has been based mainly on direct sequencing. We report here the development and validation of polymerase chain reaction-based assays for these two predominant types of *EGFR* mutations. The assay for exon 19 mutations is based on length analysis of fluorescently labeled polymerase chain reaction products, and the assay for the exon 21 L858R mutation is based on a new *Sau*96I restriction site created by this mutation. Using serial dilutions of DNAs from lung cancer cell lines harboring either exon 19 or 21 mutations, we detected these mutations in the presence of up to ~90% normal DNA. In a test set of 39 lung cancer samples, direct sequencing detected mutations in 25 cases whereas our assays were positive in 29 cases, including 4 cases in which mutations were not apparent by sequencing. These assays offer higher sensitivity and ease of scoring and eliminate the need for sequencing, providing a robust and accessible approach to the rapid identification of most lung cancer patients likely to respond to EGFR inhibitors. *(J Mol Diagn 2005, 7:396–403)*

A recent finding that is having a major impact on adult solid tumor oncology is that of somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (*EGFR*) gene in lung adenocarcinomas that respond to the EGFR inhibitor gefitinib (Iressa).[1–3] Tumors sensitive to erlotinib (Tarceva), another EGFR inhibitor with a mechanism of action similar to that of gefitinib, also contain the same types of *EGFR* mutations.[3] Given the annual incidence of lung adenocarcinomas, eg, ~70,000 in the US, the volume of molecular diagnostic assays for *EGFR* mutations could rival that of *ERBB2* amplification assays in breast cancers, if *EGFR* testing becomes part of standard lung cancer management.

Two mutations account for ~90% of *EGFR* mutations reported to date in lung adenocarcinomas (Table 1). The most common mutation type, seen in ~46% of cases with *EGFR* mutations, is a short in-frame deletion of 9, 12, 15, 18, or 24 nucleotides in exon 19. The second most common mutation, seen in ~43% of cases with *EGFR* mutations, is a point mutation (CTG to CGG) in exon 21 at nucleotide 2573, that results in substitution of leucine by arginine at codon 858 (L858R). Other much less common mutations have been described in exons 18, 20, and 21 (Table 1). Combining data from four studies,[1–4] it appears that ~80% of tumors that respond to gefitinib or erlotinib contain missense mutations or in-frame deletions in the EGFR tyrosine kinase domain, compared to none of 36 drug-refractory tumors ($P < 0.05$). These studies show that these *EGFR* mutations correlate strongly with sensitivity to specific EGFR inhibitors and that their detection could be used to predict which patients will respond to these drugs.

So far, screening for these mutations has been based on direct sequencing or single-strand conformation polymorphism analysis.[5] We report here the development and validation of polymerase chain reaction (PCR)-based assays for the two predominant *EGFR* mutations. These assays offer ease of scoring and higher analytical sensitivity and eliminate the need for sequencing. Thus, they provide a robust and accessible approach to the rapid identification of most lung cancer patients who are likely to respond to specific EGFR inhibitors.

Materials and Methods

Samples and Cell Lines

Tumor specimens were obtained through protocols approved by the Institutional Review Board of Memorial Sloan-Kettering Cancer Center. Thirty-nine lung cancer

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Table 1. Summary of Published Data on *EGFR* Mutation Types in Lung Adenocarcinomas

Reference	1	2	3	4	5	8	Total	%
Total number of *EGFR* mutated cases	$n = 10$	$n = 22$	$n = 23$	$n = 46$*	$n = 39$	$n = 111$	$n = 251$	
Exon 19 deletion	6	15	9	16	18	52	116	46.2
Exon 21 L858R	2	5	13	22	17	49	108	43.0
Exon 18 G719S or G719C	1	2	0	2†	3	4§	12	4.8
Exon 20	0	0	0	2	n.t.	5¶	7	2.8
Exon 21 non-L858R	1	0	1	4‡	1	0	7	2.8
Exon 19 insertion	0	0	0	0	0	1	1	0.4

Rare cases with a combination of a hotspot mutation (exon 19 deletion or exon 21 L858R) with a nonhotspot mutation are listed only once, under the hotspot mutation. Very rare cases with a combination of two nonhotspot mutations are listed only under one of the two mutations and further details provided in the footnotes. There are no cases reported with two different hotspot mutations.

*One case with an *EGFR* nonsense mutation not included.

†Both cases also had exon 20 mutations.

‡One case also had another exon 21 non-L858R mutation.

§One case also had an exon 18 non-G719 mutation.

¶One case had two exon 20 point mutations.

samples were studied with both assays, including all cases reported to have exon 19 or 21 mutations in our initial sequencing study that had material available for further analysis.[3] Dilutions for sensitivity studies were performed by mixing DNA extracted from positive control lung cancer cell lines NCI-H1650 or NCI-H1975 (American Type Culture Collection, Rockville, MD) into *EGFR*-germline lymphoma DNA. Each mixture contained 100 ng of total DNA with the proportion of cell line DNA ranging from 100 to 0.8%.

EGFR *Exon 19 Deletion Assay*

Because 99% of exon 19 mutations reported to date have been short in-frame deletions (Table 1), we designed an assay based on length analysis of fluorescently labeled PCR products. A 207-bp genomic fragment including all of exon 19 was amplified using primers EGFR-Ex19-FWD1 and EGFR-Ex19-REV1 (Table 2). The reverse primer was labeled with the 6-FAM fluorophore (6-FAM emits fluorescence with a peak wavelength of 522 nm). The PCR reaction mix was made up as follows: HotStarTaq DNA polymerase and 10 × buffer (Qiagen, Valencia, CA), EGFR-Ex19-FWD1 and EGFR-Ex19-REV1 primers (15 pmol each), genomic DNA template (100 ng), PCR Carry-Over prevention kit reagents (*N*-glycosylase + dUTP) (Applied Biosystems, Foster City, CA), remaining dNTPs, $MgCl_2$ (0.5 mmol/L and sterile distilled water (to 50 μl). The PCR was performed as follows: 50°C × 2 minutes (to complete the PCR Carry-Over prevention procedure), 95°C × 15 minutes (to inactivate *N*-glycosylase and activate *Taq*DNA polymerase), followed by 40 cycles of 95°C × 0.5 minutes, 60°C × 1 minute, 72°C × 1 minute, and a final extension of 72°C × 10 minutes. For DNA extracted from frozen tissue, 35 cycles was sufficient. PCR product intensity was checked on a 2% agarose gel. If PCR product intensity was strong (equal or stronger than size marker), a 1 in 50 dilution was made of which 1 μl was added into 20 μl of formamide plus 1 μl of Genescan 400HD size standard (Applied Biosystems). If the PCR product intensity was weak (band fainter than size marker), up to 1 μl of undiluted PCR product was added to 20 μl or formamide and 1 μl of Genescan 400HD size standard. The samples were denatured at 95°C for 5 minutes and cooled on ice for 5 minutes. They were then subjected to capillary electrophoresis using POP4 polymer with an excitation wavelength of 494 nm and a detection wavelength of 522 nm on an ABI 3100 Avant genetic analyzer (Applied Biosystems).

Table 2. Primers Used for Mutation Assays and Direct Sequencing

Name	Sequence
EGFR-Ex19-FWD1	GCACCATCTCACAATTGCCAGTTA
EGFR-Ex19-REV1	Fam-AAAAGGTGGGCCTGAGGTTCA
EGFR-Ex21-FWD1	CCTCACAGCAGGGTCTTCTCTGT
EGFR-Ex21-REV1	Fam-TCAGGAAAATGCTGGCTGACCTA
EGFR-Ex19-FWD-seq	CCCAGCAATATCAGCCTTAGGTG
EGFR-Ex19-REV-seq	CCACTAGAGCTGGAAAGGGAAAGA
EGFR-Ex21-FWD-seq	TCCATTCTTTGGATCAGTAGTCACTAAC
EGFR-Ex21-REV-seq	GCTCACACTACCAGGAGACCCT

All sequences are 5′ to 3′. All the primers were synthesized by Integrated DNA Technologies (Coralville, IA).

EGFR *Exon 21 L858R Mutation Assay*

The 2573T>G mutation creates a new *Sau*96I restriction site, GGNCC (Figure 1) that can be used as the basis for a PCR restriction fragment length polymorphism (PCR-RFLP) assay design. Another *Sau*96I restriction site upstream in exon 21 provides an internal restriction enzyme digestion control. The digested fluorescently labeled PCR products are analyzed by capillary electrophoresis. The precise product sizing possible by this approach allows unambiguous and sensitive identification of the 173-bp digested wild-type product and 87-bp digested mutant PCR product. Specifically, a 222-bp genomic fragment including all of exon 21 was amplified using primers EGFR-Ex21-FWD1 and EGFR-Ex21-REV1 (Table 2). Again, the reverse primer was labeled with the 6-FAM fluorophore. The PCR reaction mix was made up as above, but with EGFR-Ex21-FWD1 and EGFR-Ex21-REV1 primers (15 pmol each). The PCR was performed as follows: 50°C × 2 minutes (to complete PCR Carry-Over prevention procedure), 95°C × 15 minutes (to inactivate *N*-glycosylase and activate *Taq*DNA polymerase), followed by 95°C × 0.5 minutes, 60°C × 1 minute, 72°C ×

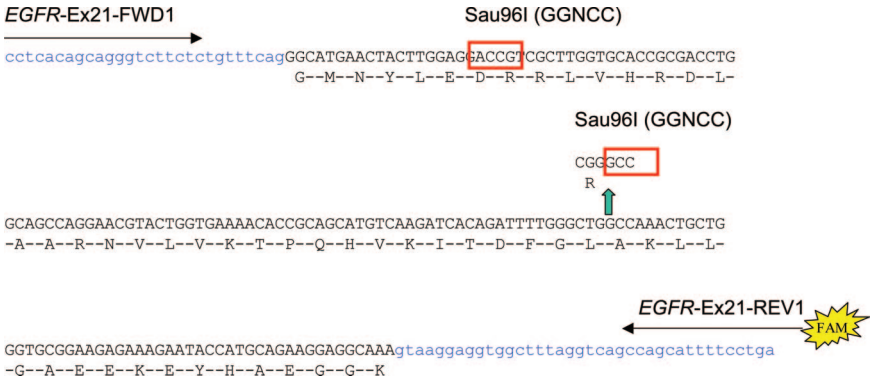

Figure 1. *EGFR* exon 21 L858R mutation assay design showing primer locations and native and mutant Sau96I restriction enzyme sites.

1 minute, and a final extension of 72°C × 10 minutes for 40 cycles if the DNA was from paraffin blocks, 35 cycles if the DNA was from frozen tissue. PCR product intensity was checked on a 2% agarose gel. The PCR products were then purified using PCR Kleen Spin column (Bio-Rad, Hercules, CA) at 735 × *g* for 2 minutes. This step was not included in the procedure for the exon 19 deletion analysis, but was found to be necessary in the exon 21 assay to reduce baseline noise and increase sensitivity after restriction enzyme digestion. The *Sau*96I digestion reaction was then performed at 37°C for 2 hours and consisted of the following: 5 μl of PCR product, 2 μl of 10× NEBuffer 4 (New England Biolabs, Beverly, MA), 2 μl (10 U) of *Sau*96I restriction enzyme (New England Biolabs), and 11 μl of sterile distilled water. After digestion, 1 μl of undiluted *Sau*96I-digested PCR product was added to 20 μl of formamide and 1 μl of Genescan 400HD size standard. The samples were denatured at 95°C for 5 minutes, cooled on ice for 5 minutes, and subjected to capillary electrophoresis as described above.

Direct Sequencing

Exons 19 and 21 were amplified using HotStarTaq DNA polymerase and primers EGFR-Ex19-FWD-seq and EGFR-Ex19-REV-seq, and EGFR-Ex21-FWD-seq and EGFR-Ex21-REV-seq, respectively. The PCR products were purified using PCR Kleen Spin columns and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer's protocol on an ABI 3100 Avant genetic analyzer running ABI Prism DNA sequence analysis software (Applied Biosystems).

Results

EGFR *Exon 19 Deletion Assay*

The assay was performed as described in Materials and Methods. We scored the test as positive when the expected product of 207 bp was accompanied by a distinct peak at 9, 12, 15, 18, or 24 bp below the normal product in the electrophoretogram. Conservatively, we only accepted mutant peaks that were greater than a minimum cutoff, designated arbitrarily at least fivefold above local background noise. Examples of 9-, 12-, 15-, and 18-bp deletions are shown in Figure 2. All cases with deletions also showed a germline product of 207 bp. Exon 19 deletion with loss of the remaining normal allele appears rare but preferential PCR amplification of the shorter deleted product could result in rare cases with a very small germline product of 207 bp. Furthermore, the nonneoplastic cells present in lung cancer samples should always provide a template for PCR amplification of the germline allele. Negative cases showed the expected germline product of 207 bp and the absence of any peak above background noise at 9, 12, 15, 18, 21, or 24 bp below the normal product in the electrophoretogram. Negative controls (including placenta and 21 lymphoma samples) consistently showed the germline product only.

The H1650 lung adenocarcinoma cell line contains an exon 19 deletion.[6] Serial dilutions of H1650 cell line DNA into normal DNA were analyzed using the exon 19 deletion assay. The exon 19 deletion was readily detected in the presence of 6.25% H1650 DNA (Figure 3). In comparison, detection of the exon 19 deletion by direct sequencing was only readily possible down to a dilution of 12.5% H1650 DNA (Supplemental Figure 1 at *http://jmd.amjpathol.org/*). We should note that because of ploidy differences between cancer cell lines and nonneoplastic cells, sensitivities based on dilutions of DNA should be viewed only as approximations of the absolute sensitivity based on dilutions of cells, however this should not affect relative comparisons of the analytical sensitivities of different techniques.

The observation that the deleted peak is approximately twice the height of the germline peak in pure H1650 DNA suggests that the deleted allele may be duplicated in this cell line. Thus the analytical sensitivities estimated using this cell line might be somewhat higher than for lung cancer cells with a nonduplicated mutant allele. Low-level amplification of *EGFR* in concert with *EGFR* mutation has been described in some tumors and another lung adenocarcinoma cell line shows high-level *EGFR* amplification and exon 19 deletion.[7] We and others[8] have also observed that many clinical samples show evidence of increased copies of the mutant allele, which in practical terms can further raise the technical sensitivity of the assay.

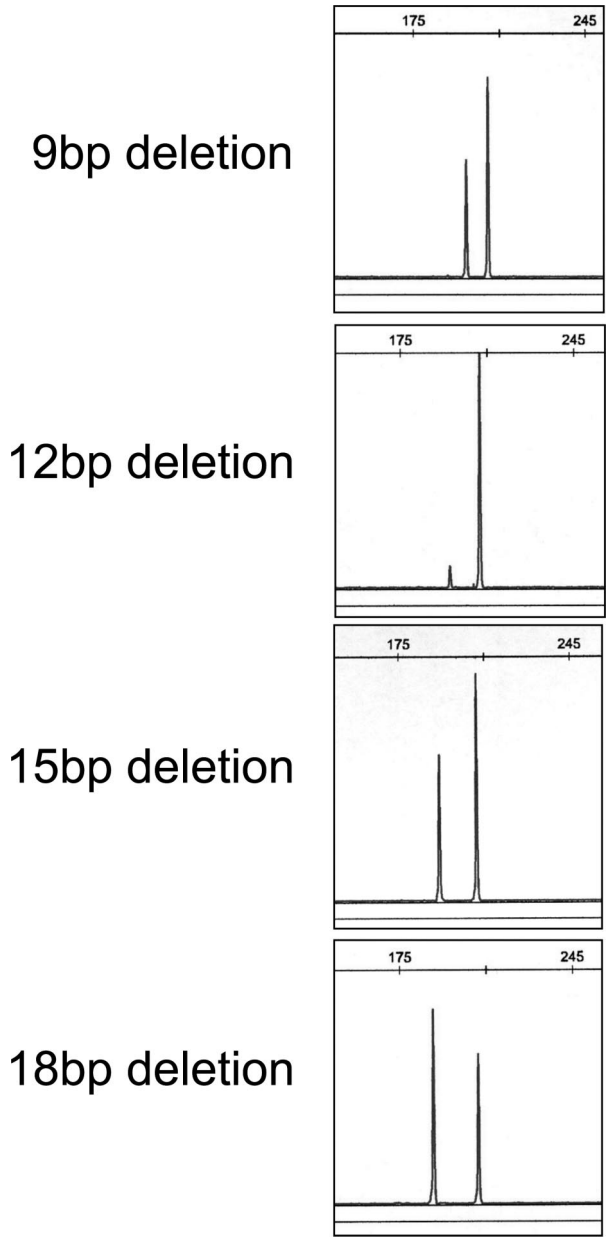

Figure 2. *EGFR* exon 19 deletion assay: examples of detection of 9-, 12-, 15-, and 18-bp deletions in *EGFR* exon 19. The variable relative heights of the deleted and germline peaks may reflect different proportions of tumor cells in these clinical tumor DNAs, or different levels of copy number alterations in the mutated *EGFR* allele.

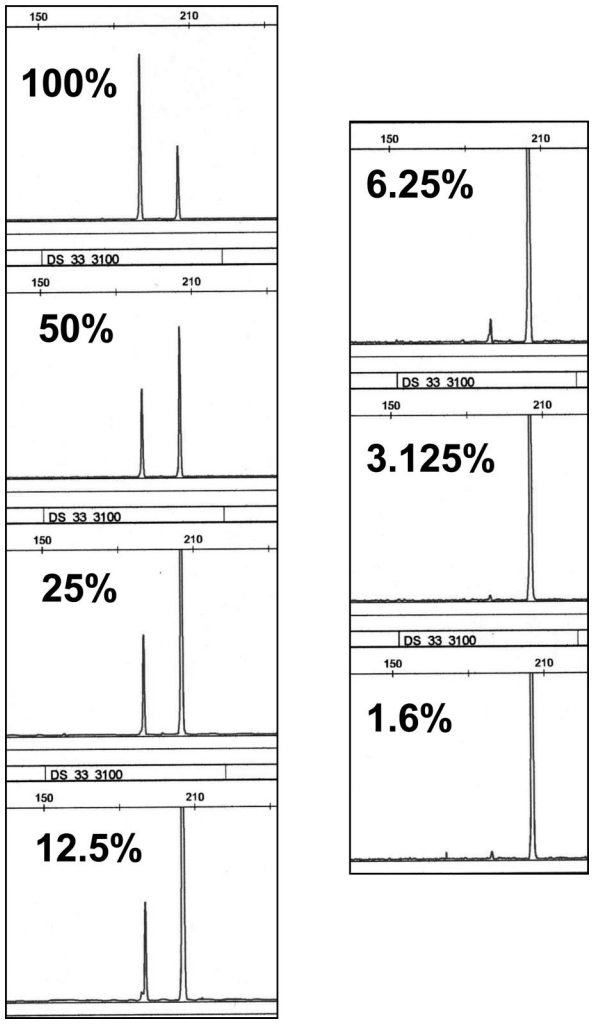

Figure 3. Sensitivity analysis of *EGFR* exon 19 deletion assay using serial dilutions of H1650 cell line DNA into normal DNA. Note that for dilutions of 25% and less the scale has been adjusted to highlight the mutant peak.

EGFR *Exon 21 L858R Mutation Assay*

The same cases were also screened for the exon 21 L858R mutation by a PCR-RFLP assay based on a new *Sau*96I restriction site created by the L858R mutation (2573T>G). The *Sau*96I-digested fluorescently labeled PCR products were analyzed by capillary electrophoresis. Examples of undigested, digested germline, and digested mutant PCR products are shown in Figure 4. All cases showed a germline product of 173 bp. Digestion of both *Sau*96I sites was complete in all runs as shown by the absence of a 222-bp undigested peak. Any case in which the peak corresponding to the mutated allele was less than five times the local background noise in the electrophoretogram (for example, see peak at 1.6% tumor cells in Figure 5) was considered at risk for a false-positive result and was repeated at least once using two to five times more template. Negative controls showed the expected germline digested product of 173 bp and the absence of any peak above background noise at 87 bp. Negative controls (including placenta and 21 lymphoma samples) consistently showed the germline product only.

The H1975 lung adenocarcinoma cell line contains the exon 21 L858R mutation.[6] Serial dilutions of H1975 cell line DNA into normal DNA were analyzed using the exon 21 L858R mutation assay. The observation that the 87-bp and 173-bp peaks are approximately equal in the presence of 100% H1975 DNA suggests that mutant and germline alleles are present in equal proportions in this cell line. The exon 21 L858R mutation was readily detected by this assay in the presence of 3.125% H1975 DNA (Figure 5). In comparison, detection of the exon 21 L858R mutation by direct sequencing was only possible

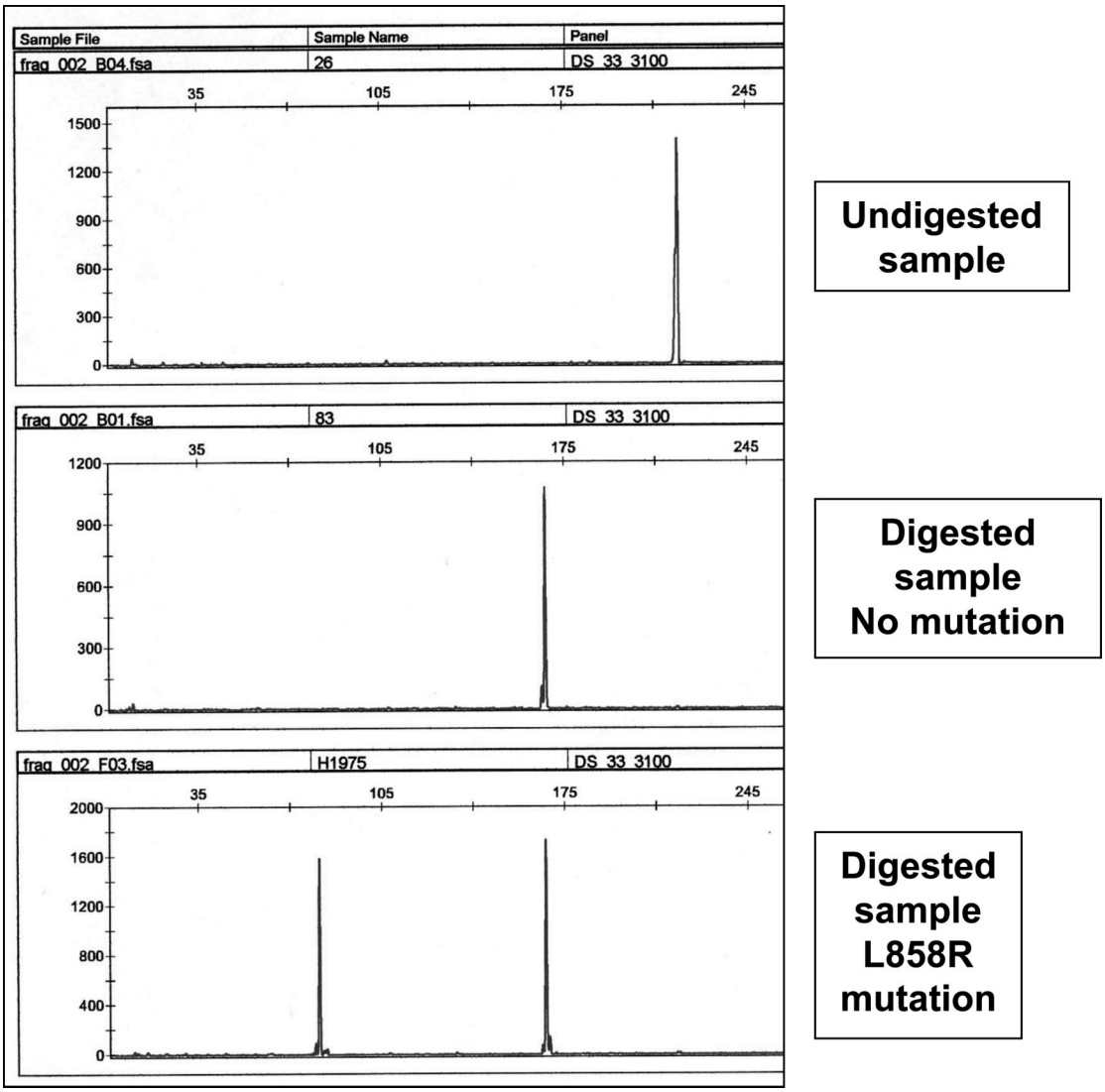

Figure 4. *EGFR* exon 21 L858R mutation assay: examples of undigested, digested germline, and digested mutant PCR products.

down to a dilution of 6.25% H1975 DNA (Supplemental Figure 2 at *http://jmd.amjpathol.org/*).

Comparison to Direct Sequencing

We then tested 39 lung cancer samples with both assays and compared the results to direct sequencing. To provide an adequate test of the sensitivity of our assays, this series of cases was enriched for mutated cases based on previous sequencing data (and is therefore not representative of mutation prevalence). The exon 19 deletion assay was positive in 15 of 39 cases and the exon 21 L858R assay was positive in 14 of 39 cases. No cases were positive for both assays. In four cases (two exon 19, two exon 21), the PCR-based assays detected mutations not apparent by direct sequencing. In an additional two cases positive by our exon 21 assay, direct sequencing was only equivocally positive (results not shown). The results are summarized in Table 3.

Discussion

Because all but 1 of the 117 exon 19 mutations reported to date have been short in-frame deletions, we designed an assay based on length analysis of fluorescently labeled PCR products on a capillary electrophoresis device. The precise PCR product sizing possible by this approach allows confirmation that the number of nucleotides deleted is a multiple of 3 as expected for in-frame deletions (Figure 2). It has been previously shown that this assay design is as accurate as and more sensitive than direct sequencing for the detection of in-frame deletion (or insertion) mutations[9] (in the context of exon 11 *KIT* mutations in gastrointestinal stromal tumors). Although there is a theoretical risk that an in-frame deletion could create a novel stop codon (with opposite biological consequences), this has not been reported in more than 100 exon 19 deletions sequenced to date. Interestingly, the only other type of mutation so far reported in exon 19,

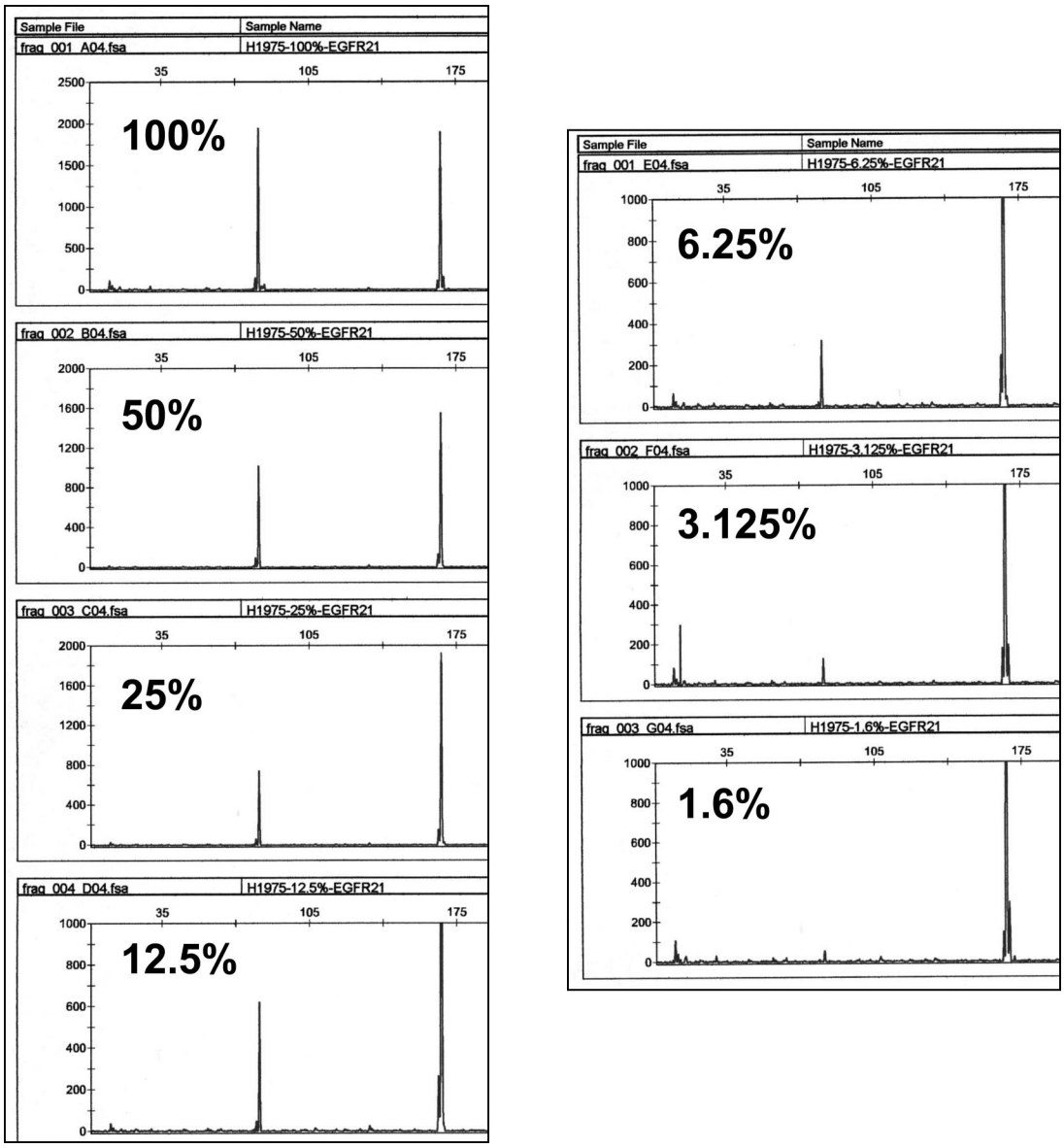

Figure 5. Sensitivity analysis of *EGFR* exon 21 L858R mutation assay using serial dilutions of H1975 cell line DNA into normal DNA. Note that for dilutions of 12.5% and less the scale has been adjusted to highlight the mutant peak.

an 18-bp insertion found in a single tumor,[8] should also be detected by the present assay. Indeed, we have recently observed another such case in our clinical testing (unpublished data).

Using this assay on ~200 clinical samples throughout a 6-month period, we have observed 9-, 12-, 15-, 18-, and 24-bp deletions in exon 19 of EGFR. By far the most common deletion size is 15 bp. A peculiar observation is that 21-bp deletions have so far not been reported and 12-bp deletions are rare. Novel deletions (eg, 6 bp, 21 bp, or 27 bp) not previously characterized in terms of function or association with therapy response should be considered of uncertain significance although their functional impact may be similar if they affect the LREA motif that forms the core of all presently described deletions. It should also be noted that, although a minority of tumors have been reported to carry two mutations (Table 1), the two hotspot mutations in exons 19 and 21 have never been found together.

Our assays found ~10% (4 of 39) more mutations than were detected by direct sequencing. Another two cases were only equivocally positive by direct sequencing (Table 3). Thus, the overall potential false-negative rate for direct sequencing compared to our assays is 15% (6 of 39), and this may be an underestimate because our study group was enriched for cases with mutations based on previous sequencing data. We attribute this to the greater analytical sensitivity of our assays. That this should be significant in the setting of lung cancer samples is not surprising given the often considerable admixture of non-neoplastic elements in these tumors.

Based on the aggregate data from four studies,[1–3,5] ~10% of lung adenocarcinomas from American or European patients who are unselected for response to EGFR

Table 3. Comparison of *EGFR* Exon 19 and 21 Mutation Status as Determined by Present PCR-Based Assays and Direct Sequencing in 39 Lung Adenocarcinomas

	EGFR exon 19		*EGFR* exon 21 L858R	
Case	Deletion assay	Direct sequencing	Mutation assay	Direct sequencing
1	15-bp deletion	nt 2235–2249 15-bp deletion	Negative	Negative
5T	Negative	Negative	Positive	Positive
12	15-bp deletion	nt 2235–2249 15-bp deletion	Negative	Negative
14	15-bp deletion	nt 2239–2256 18-bp del. + 3-bp ins.	Negative	Negative
15	Negative	Negative	Positive	Positive
18	15-bp deletion	nt 2235–2249 15-bp deletion	Negative	Negative
21	Negative	Negative	Positive	Positive
34	15-bp deletion	nt 2236–2250 15-bp deletion	Negative	Negative
65T	Negative	Negative	Positive	Positive
67	Negative	Negative	Positive	Weak Positive
76	Negative	Negative	Positive	Negative
77	12-bp deletion	Negative	Negative	Negative
79	Negative	Negative	Negative	Negative
80	Negative	Negative	Negative	Negative
83	Negative	Negative	Negative	Negative
84	Negative	Negative	Negative	Negative
85	Negative	Negative	Negative	Negative
86	Negative	Negative	Negative	Negative
87	Negative	Negative	Positive	Positive
88	Negative	Negative	Positive	Positive
89	15-bp deletion	nt 2235–2249 15-bp deletion	Negative	Negative
90	Negative	Negative	Positive	Negative
91	Negative	Negative	Negative	Negative
92	15-bp deletion	nt 2235–2249 15-bp deletion	Negative	Negative
93	Negative	Negative	Negative	Negative
94	15-bp deletion	nt 2235–2249 15-bp deletion	Negative	Negative
96	Negative	Negative	Negative	Negative
98T	Negative	Negative	Positive	Positive
99	Negative	Negative	Negative	Negative
134T	Negative	Negative	Positive	Positive
230T	15-bp deletion	nt 2236–2250 15-bp deletion	Negative	Negative
71a	Negative	Negative	Positive	Weak Positive
72a	Negative	Negative	Positive	Positive
A	9-bp deletion	nt 2238–2248 11-bp del. + 2-bp ins.	Negative	Negative
B	Negative	Negative	Positive	Positive
C	9-bp deletion	nt 2238–2248 11-bp del. + 2-bp ins.	Negative	Negative
D	9-bp deletion	Negative	Negative	Negative
E	9-bp deletion	nt 2239–2248 10-bp del. +1-bp ins.	Negative	Negative
F	18-bp deletion	nt 2239–2258 20-bp del. + 2-bp ins.	Negative	Negative

nt, nucleotide; bp, base pairs; del., deletion; ins., insertion.

inhibitors contain *EGFR* mutations. This percentage also matches the response rate of ~10% seen in single agent trials of gefitinib or erlotinib performed before the discovery of *EGFR* mutations.[10,11] However, there appear to be striking ethnic differences in the prevalence of *EGFR* mutations. It had been previously observed that responses to gefitinib were significantly more frequent in Japanese non-small cell lung cancer patients than in non-Japanese patients.[11] That unusual clinical observation is now explained by the finding of a fourfold higher prevalence of *EGFR* mutations in Asian non-small cell lung cancer patients, ie, ~40%.[4,8]

It is important to note that the use of the clinical term non-small cell lung cancer may result in unnecessary testing. This is because the vast majority of *EGFR* mutations are found in moderately to well differentiated adenocarcinomas, in particular those with partial or complete bronchioloalveolar features. The same histological features had been shown to correlate with response to EGFR inhibitors before the discovery of these mutations.[12] In contrast, mutations appear exceedingly rare in large cell carcinomas[2,5,7] and adenosquamous carcinomas.[4,8] Lastly, numerous pure squamous carcinomas (>500), including one with apparent response to gefitinib,[3] have been tested but were uniformly negative.[4,5,8]

Other strong correlates of *EGFR* mutations (and response to EGFR inhibitors) include female sex and never smoker status.[3,5,8,12] It is presently estimated that females are approximately three times as likely as males to have mutation-positive tumors and never smokers are at least five times as likely to have mutation-positive tumors as past or present smokers.[13] However, the interrelationships between these different factors have not yet been fully explored by multivariate analyses.

The two assays described here can be used as first line assays in all cases submitted for *EGFR* mutation analysis. The advantages of this overall approach to *EGFR* mutation screening is that the two types of mutation accounting for 90% of all mutations (exon 19 deletion and exon 21 L858R mutation) are detected by techniques that are faster (ie, 1 day versus 2 days) and more sensitive than direct sequencing, allowing for prompt initiation of

treatment in most of the patients likely to respond to EGFR inhibitors. If both assays are negative, these first line assays can be followed by assays based on direct sequencing of *EGFR* exons 18, 20, and 21 to detect the remaining 10% of *EGFR* mutations. The predictive value of these assays could be further enhanced by combining them with testing for *KRAS* exon 2 mutations. Recent data from our center and elsewhere have shown that *KRAS* exon 2 mutations, present in ~15 to 30% of lung adenocarcinomas, rarely, if ever, co-exist with *EGFR* mutations.[8,14] This is biologically consistent because KRAS is downstream in the EGFR signaling pathway. It is therefore not unexpected that *KRAS*-mutated lung cancers fail to respond to EGFR inhibitors, a clinically important observation.[14]

Although there is currently still no consensus on the role of *EGFR* mutation screening in patient management, it is quite possible that testing of tumor tissue for *EGFR* mutations may soon be indicated in most or all patients with moderately to well differentiated lung adenocarcinoma,[13] to aid in selecting therapy in neoadjuvant, adjuvant, and advanced/metastatic settings. In American or European centers, at least 10% of lung adenocarcinomas will harbor *EGFR* mutations and 90% of these will be detectable using the two *EGFR* mutation assays described here. Mutational analysis of *EGFR* is likely to become the mainstay of laboratory testing in this setting because other parameters such as EGFR immunoreactivity or *EGFR* gene amplification show at best only weak correlation with response to EGFR inhibitors.[7,15,16] Immunohistochemistry with antibodies to phosphorylated EGFR remains to be systematically studied in this setting but the potential of this approach is uncertain given the observation that mutant EGFR proteins generally do not show simple constitutive autophosphorylation *in vitro*.[1,3] The search for *EGFR* mutations in isolated cases of other cancers that have responded to EGFR inhibitors is in progress in many research laboratories. A wide variety of other cancers have already been screened with negative results.[13] However, it remains possible that such studies could define new indications for *EGFR* testing in clinical molecular pathology laboratories in the coming months or years.

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